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Report

GDNF Acts as a Chemoattractant to Support ephrinA-Induced Repulsion of Limb Motor Axons

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Summary

Despite the abundance of guidance cues in vertebrate nervous systems, little is known about cooperation between them [1–3]. Motor axons of the lateral motor column (LMC_L) [4, 5] require two ligand/receptor systems, ephrinA/EphA4 and glial cell line-derived neurotrophic factor (GDNF)/Ret, to project to the dorsal limb [6–8]. Deletion of either *EphA4* or *Ret* in mice leads to rerouting of a portion of LMC_L axons to the ventral limb, a phenotype enhanced in *EphA4;Ret* double mutants [7, 8]. The guidance errors in *EphA4* knockouts were attributed to the lack of repulsion from ephrinAs in the ventral mesenchyme [6, 7, 9]. However, it has remained unclear how GDNF, expressed dorsally next to the choice point [8], acts on motor axons and cooperates with ephrinAs. Here we show that GDNF induces attractive turning of LMC_L axons. When presented in countergradients, GDNF and ephrinAs cooperate in axon turning, indicating that the receptors Ret and EphA4 invoke opposite effects within the same growth cone. GDNF also acts in a permissive manner by reducing ephrinA-induced collapse and keeping the axons in a growth-competent state. This is the first example of two opposing cues promoting the same trajectory choice at an intermediate target.

Results

Glial Cell Line-Derived Neurotrophic Factor Is a Chemoattractant for LMC_L Motor Axons

The expression pattern of glial cell line-derived neurotrophic factor (GDNF) and the phenotypes of *GDNF* and *Ret* mutants [8] suggested that GDNF might have a permissive role as a growth-promoting factor or an instructive role as a chemoattractant for dorsally projecting lateral motor column (LMC_L) axons. To test whether GDNF has chemoattractive activity, we monitored the turning behavior of growth cones in response to a gradient of GDNF using the Dunn's chamber [10]. In this assay, a guidance factor is added to the outer well of the chamber, which is connected to the inner well by a narrow (20 μm) bridge (Figures 1A and 1B). Diffusion of the guidance factor leads to the formation of a stable linear gradient in the bridge ([10] and data not shown). Dissociated primary lumbar LMC neurons were prepared from embryonic day 12.5 (E12.5) *Hb9-GFP* transgenic mouse embryos in which all motor neurons and their processes are labeled with GFP [11]. All the assays were performed 17–22 hr after seeding the neurons. To test whether the identity of motor neurons is preserved in our culture conditions, we quantified the proportion of lateral LMC (LMC_L) and medial LMC (LMC_M) neurons in the culture. Because we were not able to obtain good staining of cultured cells with the available antibodies against the LMC_L marker Lim1, we instead used the LMC_M marker Islet1 [12]. After 17–22 hr in culture, the proportion of LMC_L and LMC_M cells was not different from their proportion 2 hr after plating, and the differential expression of EphA4 on the two populations [6, 7] was maintained (see Figure S1 available online).

In the control condition without any factor added to the chamber, the majority of axons continued growing in the initial direction, and the average turning angle β was near 0° (−5.2° ± 2.6°, Figures 1C–1E and Figure S1). In a linear gradient of hepatocyte growth factor (HGF), an established attractive cue for motor axons that is expressed throughout the limb [13], positive turning up the gradient was observed (24.3° ± 13.3°, Figures 1D–1F and Figure S1). When motor neurons were subjected to a gradient of GDNF, the axons also showed positive turning angles (6.6° ± 5.9° for 50 ng/ml GDNF, 11.1° ± 4.0° for 100 ng/ml GDNF, 14.4° ± 2.9° for 200 ng/ml GDNF), indicating that GDNF is a chemoattractant for LMC motor axons (Figures 1D and 1E and Figure S1). Interestingly, only axons of LMC_L neurons showed significant attractive turning, whereas the turning response of LMC_M neurons was much weaker and not significantly different from the control condition (LMC_L, mean turning angle −4.4° ± 4.8° without any cue and 19.2° ± 5.4° with GDNF, $p = 0.002$; LMC_M, mean turning angle −3.5° ± 5.2° without any cue and 9.1° ± 5.2° with GDNF, $p = 0.10$; Figures 1F and 1G).

Because Ret-independent effects of GDNF have been described in other systems [14, 15], we next asked whether the attractive activity of GDNF was Ret dependent. *Ret*-deficient neurons did not show any response to GDNF, indicating that all the chemoattractive activity of GDNF is mediated by the Ret receptor (average turning angle 13.2° ± 5.5° for $Ret^{+/-}$ and −3.6° ± 5.9° for $Ret^{-/-}$ cultures; Figure 1D and Figure S1).

We next investigated the growth-promoting properties of GDNF. GDNF was previously shown to stimulate outgrowth of axons from organotypic spinal cord cultures [16], but its effects on LMC axon growth at the sciatic plexus have not been studied in detail. Because we did not detect any difference in the speed of axonal growth in the presence and absence of GDNF in our experiments with the Dunn's chamber (data not shown), we reasoned that longer incubations were necessary for this effect to develop. In chick hindlimb, axons are known to pause for up to 24 hr at the base of the limb before exiting the dorsal-ventral (DV) choice point [17]. Because GDNF is expressed at the choice point, the axons are probably exposed to it for many hours. We therefore treated dissociated LMC cultures overnight with different concentrations of GDNF. A similar, dose-dependent increase in axon length was found in both LMC populations (Figures 1H and 1I). Therefore, GDNF has growth-promoting effects on both LMC_M and LMC_L neurons, whereas its chemoattractive activity is specific to the LMC_L population.

GDNF and ephrinAs Cooperate in Motor Axon Turning

Next we asked whether GDNF and ephrinAs cooperated in motor axon turning. As expected, preclustered ephrinA5-Fc alone caused turning away from the ephrinA source (Figure 2B and Figure S1). We then applied the two ligands in

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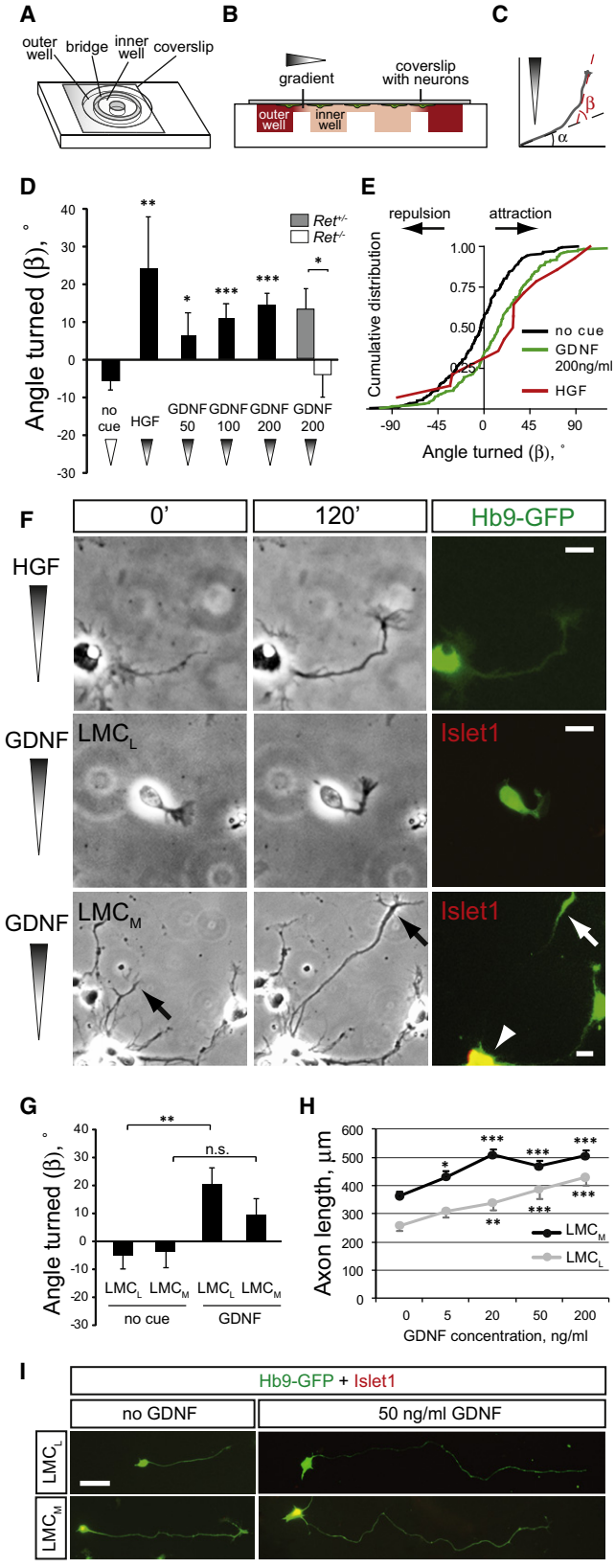

Figure 1. GDNF Acts as a Chemoattractant for LMC_L Motor Axons

(A and B) Scheme of the Dunn's chamber with a top view (A) and radial section (B).

(C) Scheme showing how the initial angle (α) and angle turned (β) were defined. β > 0 indicates attraction, β < 0 indicates repulsion.

countergradients in the Dunn's chamber to mimic the in vivo situation in which ephrinAs and GDNF are found on the opposite sides of the sciatic plexus. When 100 ng/ml GDNF was added to the inner well and 500 ng/ml preclustered ephrinA5-Fc was added to the outer well of the chamber, a turning response up the GDNF and down the ephrin gradient was observed, which was stronger than the response to either of the cues applied separately (7.5° ± 5.6° for ephrinA5, 11.1° ± 4.0° for GDNF, 25.3° ± 4.4° for ephrinA5 and GDNF in countergradients; Figures 2A and 2B and Figure S1). In contrast, no net turning was detected when GDNF and ephrinA5 were both added to the outer well (1.6° ± 4.7°; Figures 2A and 2B and Figure S1). These results indicate that GDNF and ephrinAs act simultaneously and cooperatively when presented to the axon in a spatially restricted manner, i.e., as opposing gradients. They further suggest that, in vivo, LMC_L axons would be repelled by ephrinAs in the ventral limb and at the same time attracted toward the dorsal limb by GDNF.

If this model were correct, EphA4 and Ret signaling would be unlikely to biochemically crosstalk, because this would neutralize their functions. Indeed, we could not find any evidence that the two receptors colocalized in cultured motor neurons or transfected cells or that they coclustered in response to stimulation with ephrinAs and GDNF (Figure 2C and Figure S2). Also, our attempts to demonstrate direct binding by coimmunoprecipitation were unsuccessful (Figures 2D and 2E). Moreover, the efficiency of ephrinAs in promoting growth cone collapse was not dependent on the presence of Ret (see below), and, similarly, the ability of GDNF to induce turning did not require EphA4 (average turning angle was 12.6° ± 6.7° for *EphA4*$^{+/+}$ and 14.9° ± 5.9° for *EphA4*$^{-/-}$ cultures; Figure 2F and Figure S1).

GDNF Acts as a Permissive Cue by Reducing the Repulsive Activity of ephrinAs

Because GDNF is a diffusible ligand, navigating LMC_L axons are likely to be exposed to GDNF when encountering

(D and E) Graphs showing means ± standard error of the mean (SEM) (D) and cumulative distributions (E) of turning angles (β) of LMC axons in the indicated gradients (for GDNF, numbers indicate concentration in ng/ml). The numbers of axons analyzed are: no cue, 175 axons from nine cultures; 20 ng/ml HGF, 14 axons from three cultures; 50 ng/ml GDNF, 41 axons from four cultures; 100 ng/ml GDNF, 145 axons from three cultures; 200 ng/ml GDNF, 185 axons from nine cultures; *Ret*$^{+/-}$, 49 axons from two cultures; *Ret*$^{-/-}$, 45 axons from two cultures. *p < 0.05, **p < 0.01, ***p < 0.001 (t test); all of the conditions are compared to the situation without any cue, except for *Ret*$^{-/-}$ culture.

(F) Representative examples of LMC neurons in indicated gradients taken at the beginning of the assay (0′) and at the end (120′). All images were aligned so that the gradient increases up the y axis. For GDNF gradient, coverslips were stained for Islet1 after the assay (note yellow nucleus in LMC_M neuron, arrowhead). Arrows point to the tip of the axon. Scale bars in right panels represent 20 µm.

(G) A gradient of GDNF specifically induces positive turning of LMC_L, but not LMC_M, axons. Data are presented as mean values (±SEM). The numbers of axons analyzed are: no cue, 47 LMC_L and 60 LMC_M axons from six cultures; 200 ng/ml GDNF, 48 LMC_L and 43 LMC_M axons from six cultures. These data are also included in (D) and (E). **p < 0.01; n.s. denotes not significant (t test).

(H) Quantification of axon lengths in dissociated cultures of LMC_L and LMC_M neurons treated overnight with the indicated concentrations of GDNF. Data are shown as mean values (±SEM) of >50 neurons of each population from three cultures. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the condition without GDNF (t test).

(I) Representative images of LMC_L and LMC_M neurons cultured overnight in the presence or absence of GDNF. Note that LMC_M neurons in the bottom row are positive for Islet1 (yellow nucleus). Scale bar in top left panel represents 50 µm.

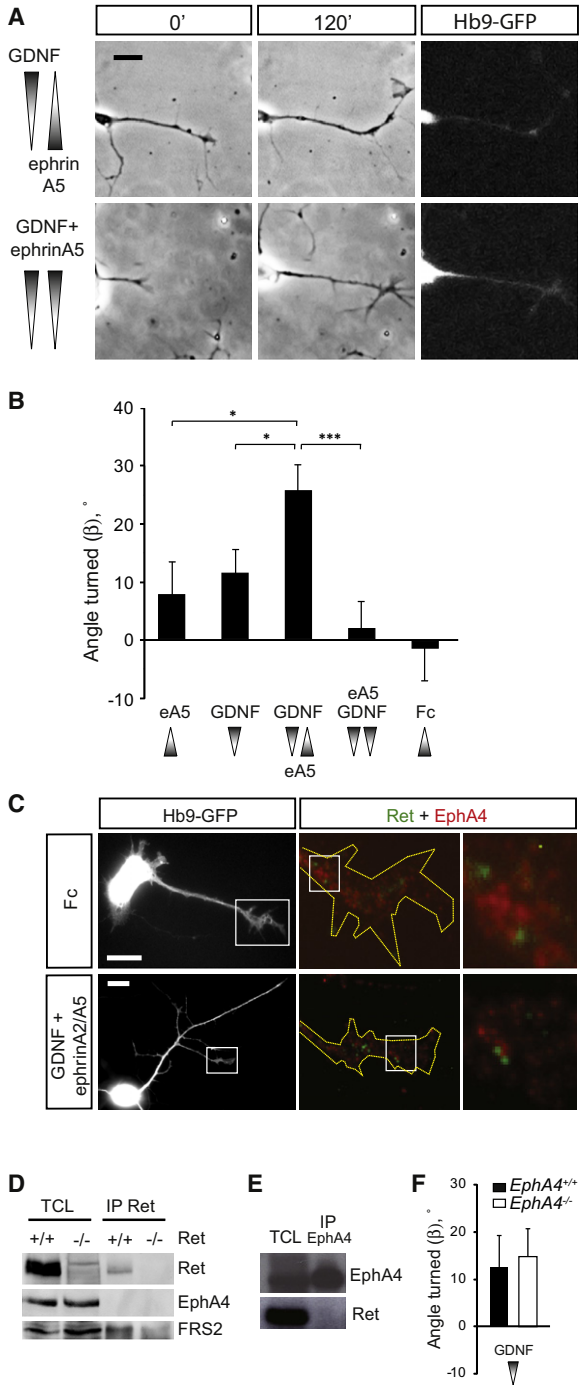

Figure 2. Cooperation between ephrinAs and GDNF in Motor Axon Turning

(A) Representative images of LMC axons exposed to GDNF and ephrinA5-Fc applied as countergradients (top) and overlapping gradients (bottom). Scale bar in top left panel represents 20 μm.

(B) Quantification of LMC axon turning in the indicated gradients. Data are presented as mean values (±SEM). The numbers of axons analyzed are: ephrinA5-Fc, 65 axons from six cultures; GDNF, 145 axons from three cultures (the same data as in Figure 1D); GDNF and ephrinA5 in countergradients, 66 axons from five cultures; GDNF and ephrinA5 in overlapping gradients, 43 axons from two cultures; Fc, 73 axons from five cultures. *$p < 0.05$, ***$p < 0.001$ (t test).

(C) Immunodetection of endogenous Ret (green) and EphA4 (red) in dissociated LMC cultures from *Hb9-GFP* transgenic embryos stimulated with the indicated proteins. For quantifications, see Figure S2. Scale bars represent 10 μm. The specificity of EphA4 and Ret antibodies was confirmed in corresponding knockout cultures (Figure S2).

(D) Lysates of E12.5 spinal cords were subjected to immunoprecipitation (IP) with the Ret antibody and examined by anti-Ret and anti-EphA4 western blots as indicated. EphA4 does not coimmunoprecipitate with Ret. As a positive control, we observed coimmunoprecipitation of Ret and FRS2, a known interaction partner of Ret [34]. TCL denotes total cell lysate.

(E) Lysates of E12.5 spinal cords were immunoprecipitated with the EphA4 antibody and examined by anti-Ret and anti-EphA4 western blots. No Ret protein is detected in the precipitates.

(F) The absence of EphA4 does not change the turning response to GDNF. Data are presented as mean values (±SEM). The numbers of axons analyzed are: $EphA4^{+/+}$, 44 axons from two cultures; $EphA4^{-/-}$, 44 axons from two cultures. The difference between wild-type and knockout cultures is not significant ($p = 0.80$, t test).

ephrinA-expressing cells of the limb mesenchyme. We therefore asked how motor axons reacted (in vitro) when the two cues, GDNF and ephrinAs, were applied in combination. Because LMC_L axons were misrouted into ephrinA-rich ventral territory in $Ret^{-/-}$ embryos in spite of maintaining a normal expression level of EphA4 [8], we hypothesized that *Ret* deficiency might render EphA4 less active. This suggested that under normal conditions, GDNF/Ret signaling might enhance ephrinA/EphA4 signaling. To test whether GDNF can modify the effects of ephrinAs on motor neurons, we cultured explants of lumbar LMC from *Hb9-GFP*$^{+}$ transgenic embryos overnight and stimulated them with different concentrations of a mixture of preclustered ephrinA2-Fc and ephrin-A5-Fc fusion proteins (the presumed endogenous ligands for EphA4 on motor axons [6]) in the presence or absence of GDNF. The application of ephrinA2-Fc and ephrin-A5-Fc caused growth cone collapse in a concentration-dependent manner (Figures 3A–3C). Simultaneous application of GDNF did not change this effect of ephrinAs (data not shown). When the cultures were treated with 50 ng/ml GDNF for 2 hr before the application of ephrinAs, in contrast to our hypothesis, the degree of growth cone collapse was reduced (Figure 3C). This effect was dependent on the GDNF concentration (Figure 3D) and required the presence of Ret (Figure 3E).

We then performed time-lapse imaging of contact events between LMC growth cones and cocultured HeLa cells expressing ephrinA5 tagged with mCherry in order to present ephrinA5 more physiologically as a cell surface-tethered cue. Although motor axons were not repelled by untransfected or mCherry-transfected control cells, every contact with an ephrinA5-expressing HeLa cell resulted in growth cone collapse. In contrast, when GDNF was added to the culture medium 1 hr before the imaging, only 72% of growth cones collapsed after these contacts (Figures 3F and 3G, Movie S1, and Movie S2). Moreover, whereas in control conditions 80% of collapsed axons retracted from the site of contact with the HeLa cell, GDNF reduced the rate of retraction to 54% (Figure 3H). GDNF also increased the growth cone recovery rate from 33% to 54% (Figure 3I) and enabled the axons to stay in contact with ephrinA5-expressing HeLa cells for longer periods of time (on average, 25 min without and 50 min with GDNF; Figure 3J). These findings indicate that GDNF reduces motor axon collapse and retraction and facilitates recovery of growth cones after ephrinA-induced collapse.

Discussion

In this study, we have shown that GDNF acts both in an instructive manner, as a chemoattractant that provides

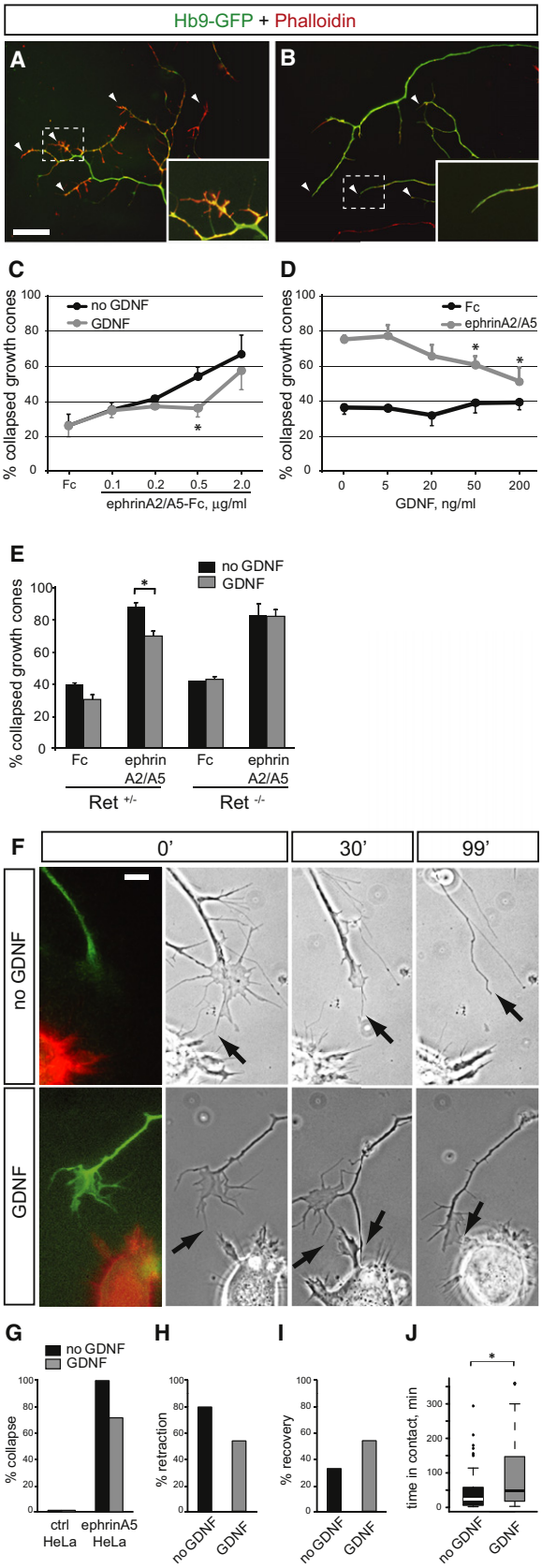

Figure 3. GDNF Reduces Repulsive Activity of ephrinAs

(A and B) Representative images of axons in *Hb9-GFP*$^{+}$ LMC explants cultured overnight and treated with preclustered Fc (A) or ephrinA2-Fc and ephrinA5-Fc (B). Arrowheads point to growth cones. Scale bar in (A) represents 50 μm. Insets in (A) and (B) are higher magnifications of the areas surrounded by the stippled boxes.

(C) Dose-response curve showing the rate of growth cone collapse in LMC explants upon application of increasing amounts of preclustered ephrinA2-Fc and ephrinA5-Fc in the presence (gray) or absence (black) of 50 ng/ml GDNF in the medium. The graph represents mean values (±SEM) from at least three cultures (in each culture, >90 growth cones in 2–4 explants were counted per condition). *$p < 0.05$ compared to the same ephrinA concentration without GDNF (t test).

(D) Dose-response curve showing the rate of growth cone collapse in LMC explants upon application of 500 ng/ml preclustered ephrinAs (gray) or preclustered Fc (black) in the presence of increasing amounts of GDNF in the medium. The graph represents mean values (±SEM) from at least three cultures (in each culture, >80 growth cones in 2–4 explants were counted per condition). *$p < 0.05$ compared to the condition without GDNF (t test).

(E) The effect of GDNF on ephrinA-treated explants is Ret dependent. Explants from *Ret* knockout embryos and control littermates were treated with 500 ng/ml preclustered ephrinA2-Fc and ephrinA5-Fc or preclustered Fc in the presence or absence of 50 ng/ml GDNF. The graph represents mean values (±SEM) from two cultures (in each culture, >100 growth cones in 2–3 explants were counted per condition). *$p < 0.05$ (t test).

(F) Representative images of time-lapse movies showing contact sites between LMC axons (green) and HeLa cells transfected with ephrinA5-mCherry (red) in the absence (top) or presence (bottom) of 50 ng/ml GDNF in the medium. Arrows point to the most distal point of the axon. Scale bar represents 10 μm.

(G–J) Quantification of growth cone collapse rate (G), axon retraction after collapse (H), growth cone recovery (I), and box plot showing the duration of contacts between growth cones and HeLa cells (J) in cocultures without (black) and with (gray) 50 ng/ml GDNF in the medium. In (G)–(I), the graphs represent the percentage of the total number of analyzed contact events that resulted in collapse, resulted in retraction, or showed recovery (no GDNF, 40 contacts from four cultures; GDNF, 39 contacts from four cultures). *$p < 0.05$ (Mann-Whitney U test).

directional information for motor axons, and in a permissive manner, by enabling fast recovery from ephrinA-induced collapse. These two functions of GDNF are likely responsible for the observed requirement of GDNF/Ret in the guidance of LMC_L axons to the dorsal limb. The chemoattractive activity also explains the redirection of ventrally fated LMC_M axons to the dorsal limb after ectopic expression of Ret in chick LMC_M neurons [8]. Our findings expand the repertoire of activities of GDNF, which has a well-established role in motor neuron survival [18]. GDNF has also previously been shown to be a chemoattractant for migrating cells, such as neuronal precursors in the rostral migratory stream and enteric neurons in the gastrointestinal tract [19, 20]. In contrast to our work, the attraction to GDNF in the rostral migratory stream was not mediated by Ret, but by NCAM [19]. The experiments with gut explants suggested that GDNF can also stimulate directed neurite outgrowth; however, they did not allow distinguishing between increased cell migration, axon outgrowth, and turning. Hence, the present study is the first demonstration that GDNF can induce rapid axon turning in a Ret-dependent manner. Moreover, the chemoattractive effect of GDNF is restricted to LMC_L neurons that express higher levels of Ret than LMC_M neurons and require GDNF/Ret signaling for path-finding in vivo. It should be noted that not all of the LMC_L axons were responsive to GDNF in our experiments. This is in agreement with the variable severity and incomplete penetrance of the *Ret* mutant phenotype [8]. A possible explanation is that GDNF might act on a subpopulation of LMC_L axons.

Our results suggest that, in addition to its function as a chemoattractant, GDNF acts as a permissive factor, keeping the growth cones at the choice point in a dynamic, growth-competent state and thereby enabling them to sample

the environment for other guidance cues. GDNF may also provide the necessary growth signal for exit of the LMC_L axons from the base of the limb, similar to the function attributed to stem cell factor (SCF) at the spinal cord midline [21]. The permissive activity of GDNF might be important to overcome the adhesive interactions within the axonal bundle and to make the dorsal territory favorable for axonal growth. In the absence of GDNF/Ret signaling, it might therefore be easier for the LMC_L axons to grow on the substrate of the ventral nerve that exits the choice point earlier than to pioneer a new pathway.

Axonal growth cones are exposed to a variety of guidance cues along their way, but little is known about how the combined action of positive and negative signals results in correct pathway choices [2, 3]. Here we describe how two opposing guidance cues, GDNF and ephrinAs, provide a "push and pull" mechanism to ensure the fidelity of the same binary pathway choice at an intermediate target. In vivo removal of either *Ret* or *EphA4* causes similar pathfinding errors of some of the axons, whereas the removal of both receptors reroutes essentially all LMC_L axons to the ventral hindlimb. To our knowledge, this is the first example of such cooperation ex vivo and in vivo. For some of the better-studied intermediate targets, several cues have been described to act simultaneously or sequentially on the same neuronal population. For example, commissural axons in the spinal cord are first attracted to the midline by Netrin and Sonic hedgehog (Shh); after midline crossing, they are repelled from the midline by Slit and Semaphorin3B (Sema3B), and finally they receive a growth impulse from SCF, which enables them to exit the floor plate. In this case, the cues regulate consecutive steps in the pathfinding process, and the phenotypes of the respective mutants are different. In the absence of Netrin or Shh signaling, commissural axons fail to reach the floor plate [22–25]. In *Slit*, *Robo1*, *Sema3B*, and *PlexinA1* mutants, they enter the floor plate but stall at the midline or recross [26, 27], whereas in *SCF* knockouts no recrossing occurs, but axons stall at the contralateral edge of the floor plate [21].

A previous study had suggested that repulsive EphA and attractive ephrinA signaling cooperated in motor axon pathfinding at the DV choice point in the limb [28], also implicating a "push and pull" mechanism like the one described here, but genetic evidence for the requirement of attractive ephrinA signaling in vivo is lacking. Similarly, in the thalamocortical system, the axons are exposed to a gradient of Netrin-1 from one side and ephrinAs from the other side; however, *Netrin-1* deletion does not exactly phenocopy the *ephrinA* and *EphA* mutants [29, 30]. Moreover, no genetic interaction has been described, and no in vitro evidence has been presented to prove cooperation between these two cues. In vitro experiments have also suggested a cooperation between an attractant (Netrin-1) and a repellant (Sema3F) in the pathfinding of the habenular nucleus axons in the diencephalon [31], but it has not been confirmed by genetic experiments. In the optic tectum, the repellant Wnt3 counterbalances ephrinB1-EphB interactions that mediate attraction [32]. In this case, however, the gradients of the repellant and attractant overlap, and single loss-of-function manipulations lead to opposite phenotypes. Instead, the cooperation between GDNF and ephrinAs is such that the two cues confront the same growth cone from opposite sides, and the absence of either receptor results in the same phenotype, which is enhanced in the absence of both receptors.

One surprising outcome of this study is that EphA4 and Ret do not seem to crosstalk to mediate turning of the growth cone. We could not detect a direct interaction between the two receptors by biochemical methods or colocalization of the two receptors by immunostaining. Moreover, in the turning assay, the outcome of applying both GDNF and ephrinAs depends on how the cues are presented spatially. We therefore believe that EphA4 and Ret, activated by their respective ligands on the different sides of the growth cones, signal independently of each other and exert opposite local effects on the cytoskeleton to promote turning. The functions of ephrinAs and GDNF at the DV choice point could thus be additive, a conclusion supported by our findings in the turning assay, where the response of motor axons to opposing gradients of GDNF and ephrinA5 amounted approximately to the sum of responses to ephrinA5 and GDNF applied individually. The spatially segregated effects of the two cues on the growth cone could also explain why GDNF, despite its permissive activity, does not seem to interfere with the ability of ephrinAs to repel LMC_L axons from the ventral mesenchyme in vivo.

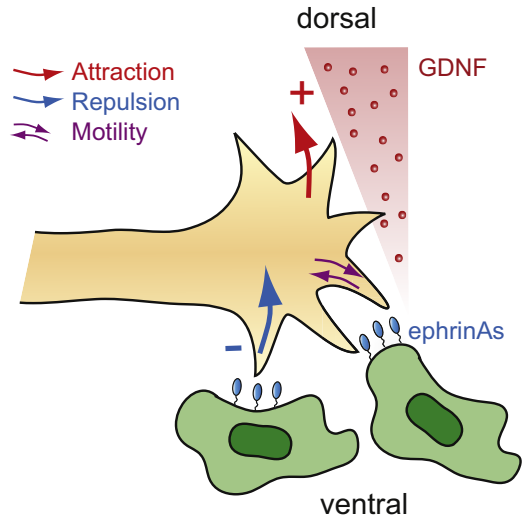

Figure 4. Model of GDNF and ephrinA Cooperation in LMC_L Axon Guidance

While a gradient of soluble GDNF attracts the axons to the dorsal side (red arrow), membrane-bound ephrinAs repel them from the ventral mesenchyme (blue arrow). In addition, GDNF maintains the motility of navigating growth cones after contact with repulsive ephrinAs (purple arrows).

In summary, our study demonstrates that GDNF serves as an instructive chemoattractant specifically for cultured LMC_L, but not LMC_M, motor neurons. When presented in opposing gradients, GDNF and ephrinAs act cooperatively. These findings suggest that, in vivo, LMC_L axons are repelled from the ventral limb by ephrinAs and at the same time attracted toward the dorsal limb by GDNF. They further support the conclusion that EphA4 and Ret signal independently and have opposite effects on the growth cone. Together with the previous genetic observations [8], this study provides the first example of two instructive and opposing cues acting simultaneously to promote the selection of the same pathway at an intermediate choice point (Figure 4). The cooperation of an attractant and a repellant in axon guidance as described in our study may be a common requirement that also applies to other axonal projections. In addition, GDNF partially suppresses growth cone collapse induced by ephrinAs. This activity prevents motor axon retraction and facilitates growth cone recovery, suggesting that, in vivo, GDNF may have a second role as a permissive cue that helps LMC_L axons to enter the dorsal limb.

Experimental Procedures

Mice

Ret knockout, *EphA4* knockout, and *Hb9-GFP* transgenic mice have been published elsewhere [8, 11, 33]. All the mutants were maintained in a comparable mixed 129/Svev × C57Bl/6 background. For knockout cultures, wild-type or heterozygous littermates were used as controls. For all other experiments with wild-type cultures, embryos were obtained from crosses of *Hb9-GFP*$^+$ males with wild-type CD1 females.

Explant and Dissociated Motor Neuron Cultures

Lower halves of the LMC (segments L3–L5) or whole LMCs were dissected from E12.5 *Hb9-GFP*$^+$ spinal cords, cut into fragments of ~100 μm, and either dissociated or cultured as explants (for details, see Supplemental Experimental Procedures). For stimulation, a 1:1 mixture of ephrinA2-Fc and ephrinA5-Fc (R&D Systems) preclustered with anti-human Fc antibodies (Jackson Immunoresearch) at a 5:1 ratio was applied for 30 min. Human IgG Fc-fragment was preclustered in the same way and used as control. For the growth cone collapse assay, GDNF (R&D Systems) was added to the culture medium 2 hr prior to ephrin stimulation. For the growth assay, GDNF was added to the cultures 1 hr after plating. For the coculture assay, HeLa cells transiently expressing ephrinA5-mCherry were detached from the plates with 0.2% EDTA in phosphate-buffered saline 36 hr after transfection and seeded onto overnight neuronal cultures. The cells were allowed to settle for 30 min before starting time-lapse imaging. Time-lapse images were acquired on an Axiovert 200M microscope (Zeiss) with a live-cell chamber using a 40× objective.

Turning Assay

Turning assay was performed with the Dunn's chamber as described [10]. For more details, see Supplemental Experimental Procedures.

Biochemistry and Immunostaining

Biochemical assays and immunostaining were performed according to standard procedures. For details, see Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes two figures, Supplemental Experimental Procedures, and two movies and can be found with this article online at doi:10.1016/j.cub.2010.11.021.

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